Polyploid Genome Structure of *Drosera spatulata* Complex (Droseraceae)

Junichi Shirakawa¹, Katsuya Nagano² and Yoshikazu Hoshi^{2*}

¹Graduate School of Bioscience, Tokai University, Kawayo, Minamiaso-mura, Aso-gun, Kumamoto 869–1404, Japan

²Department of Plant Science, School of Agriculture, Tokai University, Kawayo, Minamiaso-mura, Aso-gun, Kumamoto 869–1404, Japan

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Summary To infer genome structures and chromosome differentiations with karyomorphological changing among these 3 *Drosera* species, we applied base-specific fluorescent staining with GC-rich specific chromomycin A₃ (CMA) and AT-rich specific 4',6-diamidino-2-phenylindole (DAPI), fluorescent *in situ* hybridization (FISH) with 45S rDNA, and genomic *in situ* hybridization (GISH) with 2 parental genomic probes of *D. rotundifolia* ($2n=20$) and *D. spatulata* ($2n=40$) to somatic metaphase chromosomes of *D. tokaiensis* ($2n=60$). The chromosome ploidies in somatic cells were diploid in *D. rotundifolia*, tetraploid in *D. spatulata*, and hexaploid in *D. tokaiensis*. All 20 chromosomes of *D. rotundifolia* were middle size, while all 40 chromosomes of *D. spatulata* were small size. *Drosera tokaiensis* showed a bimodal karyotype which had 20 middle-sized chromosomes and 40 small-sized chromosomes. In base-specific fluorescent staining, satellites stained with CMA positive and DAPI negative were observed at one end of 1 pair of small sized chromosomes in *D. spatulata* and *D. tokaiensis*, but not in *D. rotundifolia*. The FISH results showed that the 45S rDNA signals of all species were located at chromosome ends or satellites. Two major signals for the 45S rDNAs were observed in *D. rotundifolia*, while 2 major signals and 2 minor signals were detected in both *D. spatulata* and *D. tokaiensis*. Dual simultaneous GISH showed the sufficient demonstration to discriminate parental genomes in *D. tokaiensis*.

Key words CMA, DAPI, Genome, *Drosera spatulata*, rDNA, FISH, GISH.

The carnivorous plant genus *Drosera* comprises nearly 150 species distributed mainly in Australia, Africa and South America, with several in the northern hemisphere (Juniper *et al.* 1989, Lowrie 1998, Rivadavia *et al.* 2003). Most of the northern hemisphere species, which belong to section *Drosera* (Seine and Barthlott 1994), are diploids with the basic chromosome number of $x=10$. In contrast, their closely related species *D. spatulata* Labill., whose distributional area is quite different from those of the northern hemisphere species, consists of a few cytotypes of different ploidy levels with $x=10$. Because of the cytotype variation, this species is often called the *Drosera spatulata* complex (Kondo 1971, Hoshi *et al.* 2008).

The *Drosera spatulata* complex, especially tetraploid lineage, has a long north-south distribution in both hemispheres from Australia, New Zealand, Indonesia, Malaysia, the Philippines, Taiwan, southern China and Japan (Merrill 1923, Van Steenis 1953, Allan 1961, Marchant and George 1982, Chen *et al.* 1984). Only in Japan, the tetraploid *D. spatulata* is sympatric with *D. rotundifolia* L., that is a geographically widespread species in the northern hemisphere. Moreover, a hexaploid lineage of *D. spatulata* complex is found only in Japan. By Nakamura and Ueda (1991), this hexaploid was treated as a distinct species *Drosera tokaiensis* (Komiya & C. Shibata) T. Nakamura & Ueda, due to having an amphidiploid state of chromosomes. Based on cytological

*Corresponding author, e-mail: yhoshi@agri.u-tokai.ac.jp

Table 1. Voucher accessions of 3 *Drosera* species sampled in this study

Species	Accession number	2n	Ploidy level (x)	Chromosome size
<i>D. rotundifolia</i>	010816Sera-1, CUK-A01, CUK-O01, KKK-R01, KKK-J-R01	20	2	Middle-sized
<i>D. spatulata</i>	JpnHa4X-3, JpnHa4X-6, JpnShinF-2-2_277'-1, KEI-01, ITK-01	40	4	Small-sized
<i>D. tokaiensis</i>	JpnHa6X-9, JpnTake5_169-1, JpnKosA-2-2_190-1, JpnKosD-5-3_223'-1, UK-01	60	6	Middle- and small-sized

and morphological studies, *D. tokaiensis* had long been considered a hybrid origin of *D. rotundifolia* and the tetraploid *D. spatulata*. As expected, the current molecular study of DNA sequencing has demonstrated that *D. rotundifolia* and the tetraploid *D. spatulata* were parents of *D. tokaiensis* (Hoshi *et al.* 2008). However, it is not clear exactly what genome- or chromosome-differentiation occurs between the 2 different genome types for forming a new *Drosera* species with hybrid origin.

Polyploidy plays a prominent role in flowering plant evolution (Leitch and Bennett 1998, Soltis and Soltis 1995, Adams and Wendel 2005). Indeed, angiosperms are estimated to be approximately 70% polyploids (Soltis and Soltis 1995, Ali *et al.* 2004), and many of these are allopolyploids, which are the forms combining the diploid nuclear genomes from 2 or more different ancestral species (Leitch and Bennett 1998). Among polyploids, in particular, allopolyploidization is thought to be an evolutionally rapid mode for new species formation from its parental species (Levy and Feldman 2002). For studying the origin and evolution of polyploids, cytogenetic techniques such as fluorescent *in situ* hybridization (FISH) using highly repeated DNA in genome have offered powerful tools. In particular, genomic *in situ* hybridization (GISH), which takes total genomic DNA as a probe, allows the unequivocal identification of allopolyploids and the visualization of their ancestral genomes. Also, GISH has been used to solve the presence of alien genomes, chromosome rearrangements, and taxonomic problems (Brysting *et al.* 2000, Refoufi *et al.* 2001, Desel *et al.* 2002, Marasek *et al.* 2004). Additionally, karyotype analysis with ribosomal DNA (rDNA) FISH has become a popular approach and has been useful for finding considerable support to clarify chromosome differentiation in each parental genome in polyploid species.

To infer genome and chromosome differentiations with karyomorphological changing among these 3 *Drosera* species, we applied fluorescent staining, FISH with 45S rDNA, and GISH with 2 parental genomic probes of *D. rotundifolia* and *D. spatulata* to somatic metaphase chromosomes of *D. tokaiensis*. The present paper shows the well demonstration to discriminate between 2 parental genomes by dual simultaneous GISH.

Materials and methods

Plant materials

Plant accessions of *Drosera rotundifolia* L., *D. spatulata* Labill. and *D. tokaiensis* (Komiya & C. Shibata) T. Nakamura & Ueda used in this study are shown in Table 1. To test intraspecific variation, 5 accessions were analyzed in each species, and no cytologically intraspecific variation was found. These plant materials were cultured on hormone-free 1/2 Murashige and Skoog basal medium (Murashige and Skoog 1962) supplemented with 0.35% gellan gum and 3% sucrose for *in vitro* culture, and maintained in the plant culture room of Department of Plant Science, School of Agriculture, Tokai University.

DNA extraction and PCR amplification

According to the method of Shaw (1988), total genomic DNAs of 3 species were isolated from young growing leaves. The isolated genomic DNAs were treated with DNase-free RNase A

(10 µg/ml) at 37°C for 1 h followed by extractions with chloroform. To track the chromosomal location of the 45S rDNA, the 18S rDNA was used as fluorescence *in situ* hybridization (FISH) probe. With extracted DNA, the 18S rDNA sequence was amplified by polymerase chain reaction (PCR) using the universal primer sets as follows: 5'-AACCTGGTTGATCCTGCCAGT-3' and 5'-TGATCCTTCTGCAGGTTACCTAC-3' for the 18S rRNA coding region. The cycle profile was an initial denaturation of 94°C (4 min), 35 cycles with 94°C (30 s), 48°C (30 s) and 72°C (60 s), and a final extension step of 72°C for 5 min.

Slide preparation

After root tips were pretreated with 0.2 mM 8-hydroxyquinoline for 2 h at 18°C, they were fixed in 70% ethanol for 1 h on ice, washed with distilled water for 1 h, and then macerated in an enzymatic mixture containing 4% Cellulase Onozuka RS (Yakult Pharmaceutical Industry Co., Ltd., Tokyo, Japan) and 2% Pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo, Japan) for 1 h at 37°C. After washing with distilled water for 1 h, root tips were placed onto glass slide, and spread with ethanol–acetic acid (3:1). The preparations were air-dried for 24 h at room temperature.

Fluorescent staining with CMA and DAPI

Chromosome preparations were stained with 25 µg/ml chromomycin A₃ (CMA) (Sigma-Aldrich Inc., MO, U.S.A.) in McIlvaine's buffer (pH 7.0) containing 5 mM MgSO₄ and 50% glycerol. These chromosome preparations stained with CMA were observed with a BV filter. Then, the slides were used for sequential 4',6-diamidino-2-phenylindole (DAPI) (Nacalai Tesque, Inc., Kyoto, Japan) staining. The slides were destained in 45% acetic acid for 30 min, dehydrated in a series of ethanol, and air-dried for 30 min. They were stained with 1 µg/ml DAPI in McIlvaine's buffer containing 50% glycerol. The chromosomes stained with DAPI were observed with a U filter.

Fluorescent in situ hybridization and simultaneous genomic in situ hybridization

The 18S rDNA fragment was biotin-labeled by random primed labeling technique (Feinberg and Vogelstein 1983) using Biotin-High Prime (Roche Applied Science, Inc., U.S.A.), following the supplier's instructions. For genomic *in situ* hybridization (GISH), the genomic probes were labeled with biotin-16-dUTP using Biotin-High Prime (Roche Applied Science, Inc., U.S.A.) for 1 parental genome, and DIG-11-dUTP using Dig-High Prime (Roche Applied Science, Inc., U.S.A.) for the another. To get fine FISH signals, chromosome preparations were necessary to treat with 250 µg/ml proteinase K (Nacalai Tesque, Inc., Kyoto, Japan) for 45 min at 37°C in a humid chamber. They were treated with 100 µg/ml RNase A (Nippon Gene Co., Ltd., Tokyo, Japan) for 1 h at 37°C in a humid chamber. After dehydration in a graded series of ethanol, a hybridization mixture containing 50% formamide, 10% dextran sulfate and DNA probes were dropped onto the slides. The preparations were sealed, denatured for 3 min at 78°C, and then incubated for 16 h at 37°C. Subsequently, the slides were rinsed in 2×SSC at 42°C for 10 min, 0.2×SSC at 42°C for 10 min, and 2×SSC/0.2% Tween20 at room temperature for 10 min twice. The slides were blocked with 5% bovine serum albumin in 2×SSC/0.2% Tween20 for 1 h at 37°C. Biotin-labeled and DIG-labeled probes were detected with streptavidin-Alexa Fluor 488 (Invitrogen, CA, U.S.A.) and anti-digoxigenin-rhodamine (Roche Applied Science, Inc., U.S.A.) in 2×SSC, respectively, for 2 h at 37°C in a humid chamber. The slides were washed in 2×SSC/0.2% Tween20 for 10 min twice, and 2×SSC for 10 min twice at room temperature. The preparations were then mounted in Vectashield mounting medium containing 1.5 µg/ml DAPI (Vector Laboratories, Inc., CA, U.S.A.). Chromosome images were taken by a digital camera (CoolSNAP: Roper Scientific, Inc., Chiba, Japan) on a microscope (Olympus BX51; Olympus, Tokyo, Japan).

Table 2. Comparison of karyotypes of 3 species *Drosera*

Species	2n	Ploidy level (x)	Total chromosome length (μm) (mean $\pm$ SD)	Average chromosome length (μm) (mean $\pm$ SD)	Inter-chromosomal asymmetry index	Number of CMA positive and DAPI negative site	Number of 45S rDNA signals
<i>D. rotundifolia</i>	20	2	44.9 $\pm$ 1.7	2.2 $\pm$ 0.1	0.10	—	2
<i>D. spatulata</i>	40	4	53.4 $\pm$ 3.3	1.4 $\pm$ 0.1	0.19	2	4
<i>D. tokaiensis</i>	60	6	87.0 $\pm$ 2.7	1.5 $\pm$ 0.2	0.57	2	4

Chromosome measurements

All *Drosera* chromosomes at mitotic metaphase could not be classified by the method using localized-centromeric position, since they do not have localized centromere and/or primary constriction (Kondo 1976, Kondo and Lavarack 1984, Kondo and Segawa 1988). Thus, individual chromosome length at mitotic metaphase was only measured. Chromosome size is defined as: middle size (*ca.* 2 μm) and small size (*ca.* 1 μm). Interchromosomal asymmetry index proposed by Romero Zarco (1986) was calculated by standard deviation of average length of chromosome complement/average length of chromosome complement.

Results and discussion

Karyotypes

Table 2 summarizes the chromosomal characteristics at mitotic metaphase of the 3 closely-related *Drosera* species studied here. The chromosome numbers of *D. rotundifolia*, *D. spatulata* and *D. tokaiensis* were 2n=20, 40 and 60, respectively. The same counts of the chromosome numbers were previously reported in these species (Hoshi and Kondo 1998). As mentioned in the previous reports, our chromosome counts supported that different ploidy levels with the same basic chromosome number of x=10 were shown in the species of section *Drosera*. Thus, the chromosome ploidies in somatic cells were the diploid in *D. rotundifolia*, tetraploid in *D. spatulata*, and hexaploid in *D. tokaiensis*.

Drosera rotundifolia and *D. spatulata* had monomodal karyotypes, showing similar chromosome size from the largest to the smallest in each species. All 20 chromosomes of *D. rotundifolia* were middle size and approximately 2 μm in length, while all 40 chromosomes of *D. spatulata* were small and approximately 1 μm . In contrast to these 2 species, *D. tokaiensis* showed a bimodal karyotype which had 20 middle-sized chromosomes of *ca.* 2 μm length each and 40 small-sized chromosomes in *ca.* 1 μm length each. The karyotype of *D. tokaiensis* showed quite strong bimodality with high asymmetry index (Table 2) and then one chromosome group could be easily distinguished from other by size, even in its most condensed stage of mid-metaphase. Generally, it suggests that most plant species with bimodal karyotypes are amphidiploid origin. Moreover, a recent molecular study of the *D. spatulata* complex with ITS and *rbcL* sequences strongly suggested that *D. tokaiensis* was an allopolyploid with hybrid origin between *D. rotundifolia* and the tetraploid *D. spatulata* (Hoshi *et al.* 2008). Therefore, genome sets and their chromosome sizes in the both parental species are well-conserved in the hexaploidal karyotype of *D. tokaiensis*.

Fluorescent staining with CMA and DAPI

In *D. tokaiensis*, 20 middle-sized chromosomes strongly fluoresced both in CMA and DAPI staining, while 40 small-sized chromosomes faintly fluoresced in CMA and strongly fluoresced in DAPI (Fig. 1). The same result was shown in previous work (Hoshi and Kondo 1998). The result suggests that the middle-sized chromosomes and the small-sized chromosomes have quite different

Fig. 1. Fluorescent staining of somatic chromosome complements of *D. tokaiensis* (a. CMA, b. DAPI). Arrowheads indicate the satellites exhibited CMA positive and DAPI negative sites. A scale bar represents 5 μ m.

DNA base compositions. Moreover, the base specific fluorescent staining pattern in the chromosome complements indicates that adenine-thymine base pair rich (AT-rich) base compositions in genome sets with 40 small-sized chromosomes are higher than those of 20 middle-sized chromosomes, since CMA and DAPI base-specific fluorescent dyes bind to guanine-cytosine base pair rich (GC-rich) and AT-rich DNA sequences, respectively (Schweizer 1976).

Satellites stained with clear CMA positive and DAPI negative (CMA⁺DAPI⁻) were observed at one end of one pair of small sized chromosomes in *D. spatulata* and *D. tokaiensis*, but not *D. rotundifolia*. In higher plants and animals, a nucleolar organizer often appears near the end of chromosome and/or satellite. An active site of the 45S rDNA, which is an essential for life activity due to a component of ribosome to produces proteins, shows a positional coincidence with nucleolar organizer region (NOR). Because 45S rDNA contains GC-rich DNA sequences, this site, especially the active NOR is stained with CMA⁺DAPI⁻. Thus, there is no doubt that active NORs in *D. spatulata* and *D. tokaiensis* are located at the CMA⁺DAPI⁻ satellites of the 2 small sized sat-chromosomes. In contrast, *D. rotundifolia* did not show any satellites. *Drosera rotundifolia*, however, must have chromosome(s) possessing NORs in the genome, since this region is a vital part of a nucleolus formation. Therefore, the NORs of *D. rotundifolia* may be at one end of one pair of middle-sized chromosomes in the somatic complement, considering satellite numbers and locations of the tetraploid and hexaploid *Drosera* species.

45S rDNA fluorescent in situ hybridization

Chromosome images with the 45S rDNA FISH signals to mitotic metaphase of the 3 *Drosera* species are shown in Fig. 2, and the signal number and its locations are summarized in Table 2. In this study, we here present the first report of 45S rDNA FISH among the 3 *Drosera* species. These 3 species were distinguishable in terms of FISH patterns of the rDNAs. The 45S rDNA signals of all species were located at chromosome ends or satellites. Two signals of the 45S rDNA probe were

Fig. 2. 45S rDNA FISH signals (arrows and arrowheads) on metaphase chromosomes in *D. rotundifolia* (a), *D. spatulata* (b) and *D. tokaiensis* (c). Arrowheads indicate major signals. A scale bar represents 5 μ m.

observed in *D. rotundifolia*.

Since 2 loci are the minimum number for diploid species, the present result is just in good agreement with the observed signal number. However, detecting the 45S rDNA loci as major FISH signals in *D. rotundifolia* is an unexpected result, because rDNA signal visualizing is generally difficult in the case of chromosomal observation without any CMA⁺DAPI⁻ signal in the genome (*e.g.*, Matsuda *et al.* 2011). It means that 45S rDNA unit or locus of *D. rotundifolia* has a quite different base composition from other plant species. In higher eukaryotes, the rDNA are present in a high copy number (Richard *et al.* 2008). In angiosperm, one repeat unit of 45S rDNA contains 3 coding regions (18S, 5.8S and 26S ribosomal subunits), 2 internal transcribed spacers (ITS1 and ITS2), an external transcribed spacer (ETS), and a nontranscribed spacer (NTS) (Rogers and Bendich 1987, Álvarez and Wendel 2003). Coding regions of rDNA are highly conserved in eukaryotic organisms (Nazar *et al.* 1976). According to our sequence data in these 3 *Drosera* species, sequence homology of the ITSs among them was greater than 90% (data not shown). Although other sequence parts generally show rather lower homology, this sequence information is still not known in genus *Drosera*. Thus, the unusual non-base specific nature of 45S rDNA locus may be due to the non-coding sequences such as ETS and NTS. Another possibility is dramatic change with insertion-deletion polymorphisms (indels) into the unit or into the locus by concerted evolution or transposable element, since indels are present in the non-coding sequence units of rDNAs in *Drosera* (Hoshi *et al.* 2008) and other carnivorous plant families (Fukushima *et al.* 2011).

In contrast to *D. rotundifolia*, 4 arrays were detected in *D. spatulata*. Because *D. spatulata* as native to Japan is tetraploid and *D. rotundifolia* is a diploid species, double the number of signals are additive. Two of the 4 signals in *D. spatulata* were major, and were often observed at stretched ends or satellites, suggesting these major signal sites were the same to CMA⁺DAPI⁻ region as active NORs. The other 2 signals were minor, and then these small signal sites might be 45S rDNA loci with non-active NORs. The signal size difference in *D. spatulata* allowed us to make the following speculations; (1) Japanese tetraploid *D. spatulata* contains allopolyploid genome composition. (2) The tetraploid *D. spatulata* arose from at least 2 putatively or existentially diploidal *D. spatulata* lineages with different genome compositions during intraspecific differentiation. (3) The genome sets of one diploid lineage have preference in 45S rRNA gene expression from those of another diploid lineage. The preference of the gene expression might be obtained after tetraploid species formation by chromosome doubling event.

In spite of being a hexaploid, 4 arrays were detected in *D. tokaiensis*. This number was the same in the case of the tetraploid *D. spatulata*. Two major and 2 minor signals were also observed in *D. tokaiensis*, but were located on 2 small-sized chromosomes and 2 middle-sized chromosomes, respectively. Thus, the signal number and size in *D. tokaiensis* suggested that the major signal and minor signal sites were active and non-active NORs, respectively. The same signal numbers of 45S rDNAs in the tetraploid and hexaploid species are unexpectedly nonadditive.

Although a high copy number of rDNAs is normally clustered as tandem repeats at one or more chromosomal sites (Rogers and Bendich 1987, Álvarez and Wendel 2003), the copy number and chromosomal location of rDNAs can be rapidly changed in plant genomes (Schubert and Wobus 1985, Raina and Mukai 1999). Additionally, some examples of intraspecific variation with size changing of the 45S rDNAs are known, and are due to mechanisms such as unequal crossing over (Butler and Metzenberg 1989, Komma and Atwood 1994). Therefore, our rDNA FISH result indicates that the nonadditive number of signal sites in the hexaploid species is due to a low copy number or the absence of the 45S rDNA repeat by elimination from the 2 sets of genomes all composed of small-sized chromosomes in 1 diploid *D. spatulata* lineage. The deleting event of 45S rDNAs may occur during or after hexaploid species formation by chromosome doubling of natural triploid hybrid between the diploid and tetraploid parental species.

Simultaneous genomic in situ hybridization

Figure 3 shows metaphase chromosomes of *D. tokaiensis* after simultaneous GISH. By the simultaneous GISH, the middle-sized chromosomes clearly showed red fluorescence with rhodamine-labeled DNA from *D. rotundifolia*, while the remaining small-sized chromosomes showed green fluorescence of biotin-labeled DNA detected with Alexa Flora 488. In our GISH study of *Drosera*, the previous report only took a total genomic DNA probe of *D. rotundifolia* (Hoshi *et al.* 1994). In contrast, the present simultaneous GISH dealt with probes of both parental species, and performed distinct 2-color separation between both parental genomes. GISH results with 45rDNA loci are summarized in Fig. 4. Here we finally confirmed that 4 sets of genomes with small-sized chromosomes were derived from *D. spatulata*, and 2 sets of genomes with middle-sized chromosomes were derived from *D. rotundifolia*.

Recent cytogenetic and molecular phylogenetic studies gave considerable support to the possibility of genome size and chromosome size increases on genome evolution in this *Drosera* group. Moreover, current random amplified polymorphic DNA (RAPD) work in *Drosera* suggested that the DNA fragments obtained by RAPD primers were preferentially amplified from the genomes with 20 middle-sized chromosomes in *D. rotundifolia* and *D. tokaiensis* (Hoshi *et al.* 2010). This amplifying preference led us to predict that RAPD-generated DNA fragments contained many of the DNA sequences related to chromosomal differentiation, especially directly-linked to chromosome size changing.

The DNA sequences to change chromosome size may disperse uniformly on all middle-sized chromosomes, because each chromosome of *D. rotundifolia* genome is similar in size. The uniform accumulating of such DNA sequences into the genome seems to induce chromosome paring inhibition during meiosis, facilitating a reproductive isolation at the cytogenetic level. Even though the DNAs are non coding sequences, this speculation generates an additional idea that the uniformly dispersed DNA sequences play an important role for plant speciation or new species formation, as well as allopolyploidization in the *D. spatulata* complex.

Further studies using FISH technique with different molecular markers such as RAPD-generated repetitive sequences are necessary to clarify genome size changing and chromosome differentiation of the *Drosera spatulata* complex and its related species.

Fig. 3. GISH in metaphase chromosomes of *D. tokaiensis*. The chromosomes were counter-stained with DAPI (a), hybridized with total genomic DNA probe of *D. rotundifolia* (b) and total genomic DNA probe of *D. spatulata* (c). Overlay chromosome images were superimposed (d). A scale bar represents 5 μm .

Fig. 4. Summary of 45S rDNA FISH and simultaneous GISH results in *D. tokaiensis* and it related species.

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References

- Adams, K. L. and Wendel, J. F. 2005. Polyploidy and genome evolution in plants. *Curr. Opin. Plant Biol.* **8**: 135–141.
- Ali, H. B. M., Lysak, M. A. and Schubert, I. 2004. Genomic *in situ* hybridization in plants with small genomes is feasible and elucidates the chromosomal parentage in interspecific *Arabidopsis* hybrids. *Genome* **40**: 954–960.
- Allan, H. H. 1961. Droseraceae. In H. H. Allan (ed.). *Flora of New Zealand* **1**: 200–203.
- Álvarez, I. and Wendel, J. F. 2003. Ribosomal ITS sequences and plant phylogenetic inference. *Mol. Phylogenet. Evol.* **29**: 417–434.
- Brysting, A. K., Holst-Jensen, A. and Leitch, I. 2000. Genomic organization of the hybrid *Poa jemtlandica* (Poaceae) verified by genomic *in situ* hybridization and chloroplast DNA sequences. *Ann. Bot.* **85**: 439–445.
- Butler, D. K. and Metzenberg, R. L. 1989. Premeiotic change of nucleolus organizer size in *Neurospora*. *Genetics* **122**: 783–791.
- Chen, W., Ruan, Y., Fu, S. and Fu, K. 1984. Droseraceae. *Flora Reipublicae Popularis Sinae* **34**: 14–30.
- Desel, C., Jansen, R., Dedong, G. and Schmidt, T. 2002. Painting of parental chromatin in beta hybrids by multi-color fluorescent *in situ* hybridization. *Ann. Bot.* **89**: 171–181.
- Feinberg, A. P. and Vogelstein, B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to High specific activity. *Anal. Biochem.* **132**: 6–13.
- Fukushima, K., Imamura, K., Nagano, K. and Hoshi, Y. 2011. Contrasting patterns of the 5S and 45S rDNA evolutions in the *Byblis liniflora* complex (Byblidaceae). *J. Plant Res.* **124**: 231–244.
- Hoshi, Y., Hizume, M. and Kondo, K. 1994. Genomic *in situ* hybridization to improve a hypothesis on natural-hybrid origin of the hexaploid *Drosera spatulata* 'Kansia type'. *La Kromosomo II* **75-76**: 2619–2623.
- and Kondo, K. 1998. Chromosome differentiation in *Drosera*, Subgenus *Rorella*, Section *Rossolis*. *Cytologia* **63**: 199–211.
- , Shirakawa, J., Hasebe, M., Fukushima, K. and Kondo, K. 2008. Tandem repeat rDNA sequences derived from parents were stably maintained in hexaploids of *Drosera spatulata* complex (Droseraceae). *Cytologia* **73**: 313–325.
- , —, Takeo, M. and Nagano, K. 2010. A molecular genetics of *Drosera spatulata* complex by using of RAPD analysis. *Chromosome Bot.* **5**: 23–26.
- Juniper, B. E., Robins, R. J. and Joel, D. M. 1989. *The carnivorous plants*. Academic Press. London.
- Komma, D. J. and Atwood, K. C. 1994. Magnification in *Drosophila*: evidence for an inducible rDNA-specific recombination system. *Mol. Gen. Genet.* **242**: 321–326.
- Kondo, K. 1971. A review of the *Drosera spatulata* complex. *Shokubutsu Kenkyu Zasshi* **46**: 321–326.
- 1976. A cytotaxonomic study in some species of *Drosera*. *Rhodora* **78**: 532–541.
- and Lavarack, P. S. 1984. A cytotaxonomic study of some Australian species of *Drosera* L. (Droseraceae). *Bot. J. Linn. Soc.* **88**: 317–333.
- and Segawa, M. 1988. A cytotaxonomic study in artificial hybrids between *Drosera anglica* Huds. and its certain closely related species in series *Drosera*, section *Drosera*, subgenus *Drosera*, *Drosera*. *La Kromosomo II* **51-52**: 1697–1709.
- Leitch, I. J. and Bennett, M. D. 1998. Polyploidy in angiosperms. *Trends Plant Sci.* **2**: 470–476.
- Levy, A. A. and Feldman, M. 2002. The impact of polyploidy on grass genome evolution. *Plant Physiol.* **130**: 1587–1593.
- Lowrie, A. 1998. *Carnivorous plants of Australia*, vol. 3. University of Western Australia Press, Western Australia.
- Marchant, N. G. and George, A. S. 1982. *Drosera*. In: *Flora of Australia*, vol. **8**: 9–64. Australian Government Publishing Service, Canberra.
- Marasek, A., Hasterok, R., Wiejacha, K. and Orlikowska, T. 2004. Determination by GISH and FISH of hybrid status in *Lilium*. *Hereditas* **140**: 1–7.
- Matsuda, Y., Kaneko, H., Murata, T., Nagano, K. and Hoshi, Y. 2011. A cytogenetic study of *Polytrias amaura* (Poaceae). *Chromosome Bot.* **6**: 5–11.
- Merrill, E. D. 1923. *An Enumeration of Philippine Flowering Plants*, Vol. 2. Bureau of Printing, Manila.
- Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**: 473–497.
- Nakamura, T. and Ueda, K. 1991. Phytogeography of Tōkai hilly land element II. Taxonomic study of *Drosera tokaiensis* (Komiya & C. Shibata) T. Nakamura & Ueda (Droseraceae). *AP6* **42**: 125–137.
- Nazar, R. N., Sitz, T. O. and Busch, H. 1976. Sequence homologies in mammalian 5.8S ribosomal RNA. *Biochemistry* **15**:

- 505–508.
- Raina, S. N. and Mukai, Y. 1999. Detection of a variable number of 18S-5.8S-26S and 5S ribosomal DNA loci by fluorescent *in situ* hybridization in diploid and tetraploid *Arachis* species. *Genome* **42**: 52–59.
- Refoufi, A., Jahier, J. and Esnault, M. A. 2001. Genomic analysis of *Elytrigia pycnantha* and *Thinopyrum junceiforme* and of their putative natural hybrid using the GISH technique. *Genome* **44**: 708–715.
- Richard, G. F., Kerrest, A., Dujon, B. 2008. Comparative genomics and molecular dynamics of DNA repeats in eukaryotes. *Microbiol. Mol. Biol. Rev.* **72**: 686–727.
- Rivadavia, F., Kondo, K., Kato, M. and Hasebe, M. 2003. Phylogeny of the sundews, *Drosera* (Droseraceae), based on chloroplast *rbcL* and nuclear 18S ribosomal DNA sequences. *Am. J. Bot.* **90**: 123–130.
- Rogers, S. O. and Bendich, A. J. 1987. Ribosomal RNA genes in plants: variability in copy number and in the intergenic spacer. *Plant Mol. Biol.* **9**: 509–520.
- Romero Zarco, C. 1986. A new method for estimating karyotype asymmetry. *Taxon* **35**: 526–530.
- Schweizer, D. 1976. Reverse fluorescent chromosome banding with chromomycin and DAPI. *Chromosoma* **58**: 307–324.
- Schubert, I. and Wobus, U. 1985. *In situ* hybridization confirms jumping nucleolus organizing regions in *Allium*. *Chromosoma* **92**: 143–148.
- Seine, R. and Barthlott, W. 1994. Some proposals on the infrageneric classification of *Drosera* L. *Taxon* **43**: 583–589.
- Shaw, C. H. 1988. Plant molecular biology. A practical approach. Oxford University Press, Oxford.
- Soltis, P. S. and Soltis, D. E. 1995. The role of genetic and genomic attributes in the success of polyploids. *Proc. Natl. Acad. Sci. U.S.A.* **97**: 7051–7057.
- Van Steenis C. G. G. V. 1953. Droseraceae. *Flora Malesiana, Series I*: 377–380.
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